Supplementary Table 1. Investigated *RUNX1* variants

Chr.21 (GRCh38)	RUNX1b (NM_001001890.3)		RUNX1c (NM_001754.5)	
genomic location	nucleotide change	protein change	nucleotide change	protein change
g.34887027	c.86T>C	p.(Leu29Ser)	c.167T>C	p.(Leu56Ser)
g.34880568	c.416G>A	p.(Arg139Gln)	c.497G>A	p.(Arg166Gln)
g.34886941	c.172C>A	p.(His58Asn)	c.253C>A	p.(His85Asn)
g.34886914	c.199A>C	p.(Ser67Arg)	c.280A>C	p.(Ser94Arg)
g.34886875	c.238C>A	p.(Arg80Ser)	c.319C>A	p.(Arg107Ser)
g.34880713	c.271_283del	p.(Val91Glyfs*11)	c.352_364del	p.(Val118Glyfs*11)
g.34880698	c.286G>C	p.(Asp96His)	c.367G>C	p.(Asp123His)
g.34880644	c.340T>G	p.(Ser114Ala)	c.421T>G	p.(Ser141Ala)
g.34880557	c.427G>C	p.(Gly143Arg)	c.508G>C	p.(Gly170Arg)
g.34834521	c.613C>T	p.(Arg205Trp)	c.694C>T	p.(Arg232Trp)
g.34834482	c.652C>T	p.(Pro218Ser)	c.733C>T	p.(Pro245Ser)
g.34834466	c.668G>A	p.(Arg223His)	c.749G>A	p.(Arg250His)
g.34799412	c.775C>A	p.(Gln259Lys)	c.856C>A	p.(Gln286Lys)
g.34880569	c.415C>T	p.(Arg139*)	c.496C>T	p.(Arg166*)
g.34859486	c.520C>T	p.(Arg174*)	c.601C>T	p.(Arg201*)
g.34834431	c.703C>T	p.(Gln235*)	c.784C>T	p.(Gln262*)
g.34792575	c.922C>T	p.(Gln308*)	c.1003C>T	p.(Gln335*)
g.34792415	c.1082C>A	p.(Ser361*)	c.1163C>A	p.(Ser388*)
g.34792314	c.1183G>T	p.(Glu395*)	c.1264G>T	p.(Glu422*)
g.34792158	c.1339G>T	p.(Glu447*)	c.1420G>T	p.(Glu474*)

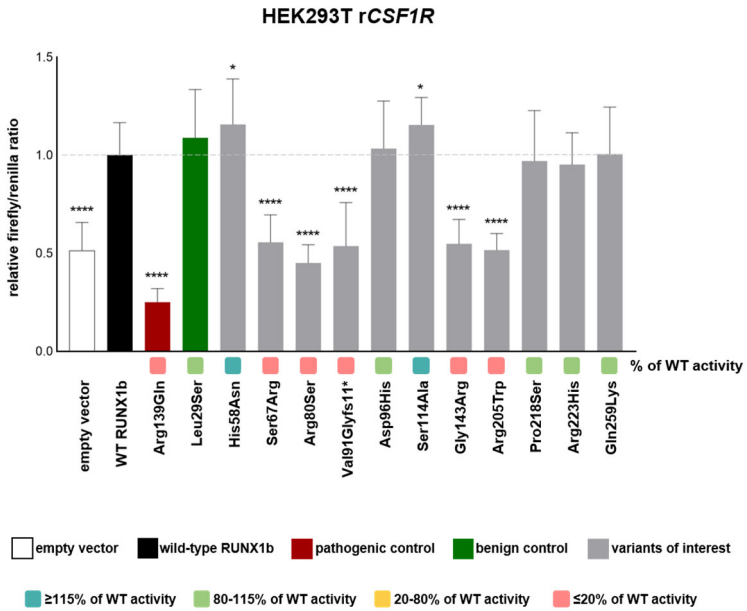
Supplementary Table 2. Primer sequences

variant	sequence	oligo name
Leu29Ser	AGATGAGCGAGGCGTCGCCGCTGGGCGCCCC	RUNX1_sdm86_f
	GGGGCGCCCAGCGGCGACGCCTCGCTCATCT	RUNX1_sdm86_r
His58Asn	GTGCTGGCCGACAACCCGGGCGAGC	RUNX1b_172C>A_fw
	GCTCGCCCGGGTTGTCGGCCAGCAC	RUNX1b_172C>A_rev
Ser67Arg	GGTGCGCACCGACCGCCCCAACTTCC	RUNX1b_199A>C_fw
	GGAAGTTGGGGCGGTCGGTGCGCACC	RUNX1b_199A>C_rev
Arg80Ser	CTGCCTACGCACTGGAGCTGCAACAAGACC	RUNX1_sdm_c.238C>A_f
	GGTCTTGTTGCAGCTCCAGTGCGTAGGCAG	RUNX1_sdm_c.238C>A_r
Val91Glyfs*11	CGCTTTCAAGGGGATGTTCCAGATGGCACTCTGGTCACTGTGATGGC	RUNX1_sdm_ c.271_283del_fw
	GGAACATCCCCTTGAAAGCGATGGGCAGGGTCTTGTTGCAGCG	RUNX1_sdm_ c.271_283del_rev
Asp96His	GGTGGCCCTAGGGCATGTTCCAGATGGC	RUNX1b_sdm_286G>C_f
	GCCATCTGGAACATGCCCTAGGGCCACC	RUNX1b_sdm_286G>C_r
Ser114Ala	GCAATGATGAAAACTACGCGGCTGAGCTGAGAAATGC	RUNX1b_340T>G_fw
	GCATTTCTCAGCTCAGCCGCGTAGTTTTCATCATTGC	RUNX1b_340T>G_rev
Arg139*	CCTCAGGTTTGTCGGTTGAAGTGGAAGAGGGAA	RUNX1_sdm415_f
	TTCCCTCTTCCACTTCAACCGACAAACCTGAGG	RUNX1_sdm415_r
Arg139Gln	CCTCAGGTTTGTCGGTCAAAGTGGAAGAGGGAA	RUNX1_sdm416_f
	TTCCCTCTTCCACTTTGACCGACAAACCTGAGG	RUNX1_sdm416_r
Gly143Arg	CGGTCGAAGTGGAAGACGGAAAAGCTTCACTCTG	RUNX1_sdm_c.427G>C_fw1
	CAGAGTGAAGCTTTTCCGTCTTCCACTTCGACCG	RUNX1_sdm_c.427G>C_rev1
Arg174*	CAGTGGATGGGCCCTGAGAACCTCGAAGA	RUNX1_C520T_fw
	TCTTCGAGGTTCTCAGGGCCCATCCACTG	RUNX1_C520T_rev
Arg205Trp	CTGGAGCAGCTGTGGCGCACAGCCATG	RUNX1b_sdm_613C>T_f
	CATGGCTGTGCGCCACAGCTGCTCCAG	RUNX1b_sdm_613C>T_r
Pro218Ser	CACCACCCAGCCTCCACGCCCAACC	RUNX1b_sdm_c.652C>T_f
	GGTTGGGCGTGGAGGCTGGGTGGTG	RUNX1b_sdm_c.652C>T_r
Arg223His	CGCCCAACCCTCATGCCTCCCTGAACCA	RUNX1b_sdm_668G>A_f
	TGGTTCAGGGAGGCATGAGGGTTGGGCG	RUNX1b_sdm_668G>A_r
Gln235*	CCACTGCCTTTAACCCTTAGCCTCAGAGTCAGATGC	RUNX1b_sdm_703C>T_f
	GCATCTGACTCTGAGGCTAAGGGTTAAAGGCAGTGG	RUNX1b_sdm_703C>T_r
Gln259Lys	CCTACGATCAGTCCTACAAATACCTGGGATCCATTG	RUNX1_sdm_c.775C>A_fw
	CAATGGATCCCAGGTATTTGTAGGACTGATCGTAGGAC	RUNX1_sdm_c.775C>A_rev
Gln308*	GCGACCCGCGCTAGTTCCCCGCGC	RUNX1b_sdm_c.922C>T_f
	GCGCGGGGAACTGGCGCGGGTCGC	RUNX1b_sdm_c. c.922C>T_r
Ser361*	CCCTACCCCGGCTAGTCGCAAGCGC	RUNX1b_sdm_c.1082C>A_f
	GCGCTTGCGACTAGCCGGGGTAGGG	RUNX1b_sdm_c.1082C>A_r
Glu395*	CATGGTGGGCGGCTAGCGCTCGCCGCC	RUNX1_sdm_c.1183G>T_fw
	GGCGGCGAGCGCTAGCCGCCCACCATG	RUNX1_sdm_c.1183G>T_rev
Glu447*	CCTCCGCGCGCCTGGAGTAGGCCGTGTGGAGGCC	RUNX1b_sdm_1339G>T_f
	GGCCTCCACACGGCCTACTCCAGGCGCGCGGAGG	RUNX1b_sdm_1339G>T_r

Supplementary Table 3. Variant classification following present classification guidelines

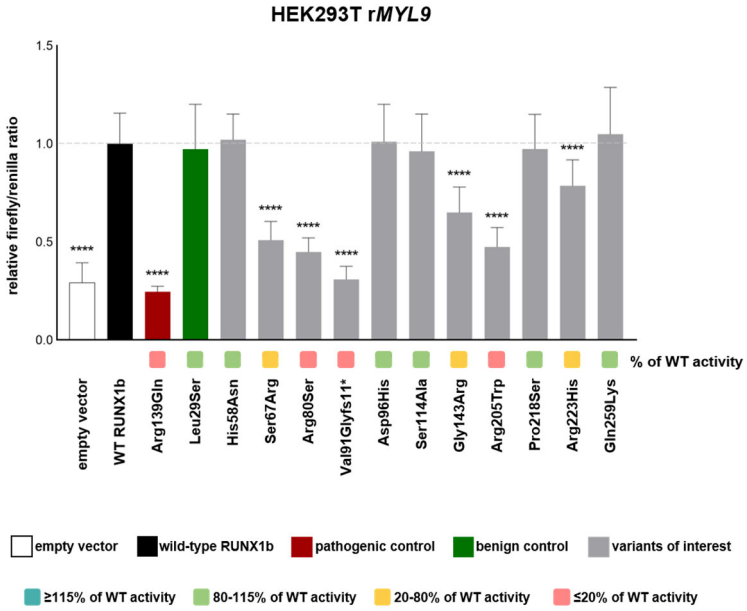
RUNX1b (NM_001001890.3)		gnomAD max. frequency	REVEL score	ClinVar ID RCV associated with RUNX1-FPD	ACMG/AMP [1] + MM-VCEP criteria [2]	func. criteria our assays	func.criteria incl. literature	classification w/o func. criteria	classification w/ func. criteria	report of germline variant carriers in literature	reference to Fig. 2, applied genetic testing approach
protein change	cDNA change										
p.(His58Asn)	c.172C>A	0.000439 (EA)	0.8519	RCV000549373.5 1xVUS (Invitae)[¥] 1xLB (MM-VCEP)	BS1 strong PP3 supporting	PS3 supporting	uncertain function[§] PMID 23817177 [3] (normal TA, DNA binding, dimerization)	VUS	VUS	PMID 34233450 [4]	
p.(Ser67Arg)	c.199A>C		0.947		PS4 supporting PM2 moderate PP3 supporting	PS3 moderate	PS3 strong PMID 23817177 [3] and PMID: 17290219 [5] (dimerization defect)	VUS	likely pathogenic	PMID 34233450 [4]	
p.(Ser67Arg)	c.201C>G		0.911		PS4 supporting PS1 moderate (c.199A>C) PM2 moderate PP3 supporting	PS3 moderate	PS3 strong PMID 23817177 [3] and PMID: 17290219 [5] (dimerization defect)	likely pathogenic	likely pathogenic		Fig. 2A Custom NGS panel [a]; NGS-based copy number analysis
p.(Arg80Ser)	c.238C>A		0.921	RCV001062338.2 VUS (Invitae) [¥]	PS4 moderate (incl. ClinVar) PM1 moderate PM2 moderate PP3 supporting PM5 supporting (Arg80His)	PS3 moderate	PS3 moderate	likely pathogenic	likely pathogenic		Fig. 2B *Trusight Myeloid* NGS panel (Illumina); no copy number analysis performed
p.(Asp96His)	c.286G>C	0.00002639 (NFE)	0.943		PM1 supporting PP3 supporting	BS3 supporting	BS3 supporting	VUS	VUS	PMID 34233450 [4]	Fig. 2D WES; NGS-based copy number analysis
p.(Val91Glyfs*11)	c.271_283del				PVS1 PS4 supporting PM2 moderate	(PS3 moderate)[#]	(PS3 strong)[#] PMID 10508512 [6] (CFU defect)	pathogenic	pathogenic	PMID 10508512 [6]	
p.(Ser114Ala)	c.340T>G	0.00001549 (NFE)	0.851	RCV000685769.4 VUS (Invitae) [¥]	PM1 supporting PP3 supporting	BS3 supporting	BS3 supporting	VUS	VUS	PMID 32315381 [7] PMID 34233450 [4]	
p.(Gly143Arg)	c.427G>C		0.947		PS4 supporting PS1 moderate (c.427G>A) PM1 moderate PM2 moderate PP3 supporting	uncertain function, some evidence for TA defect	uncertain function, no secondary assays from the literature integrated, because of other/missing nucleotide change in case of a variant which might affect splicing	likely pathogenic	likely pathogenic	PMID 22430633 [8] (different nucleotide change) PMID: 24732596 [9] (nucleotide change not reported)	
p.(Arg205Trp)	c.613C>T		0.796		PS4 supporting PM2 moderate PP3 supporting	PS3 moderate	PS3 moderate	VUS	likely pathogenic		Fig. 2C NGS-based sequencing of *RUNX1*; no copy number analysis
p.(Pro218Ser)	c.652C>T		0.444	RCV000707170.2 VUS (Invitae) [¥]	PS4 moderate (incl. ClinVar) PM2 moderate	BS3 supporting	BS3 supporting	VUS	VUS	PMID 29146883 [10] PMID 26316320 [11]	Fig. 2E NGS panels (i) for myeloid malignancies [b] and (ii) for thrombocytopenia [c]; SNP array for copy number analysis
p.(Arg223His)	c.668G>A	0.00007028 (NFE)	0.488			BS3 supporting	BS3 strong PMID:23817177 [3] (normal TA, DNA binding, dimerization)	VUS	VUS	PMID 34233450 [4] PMID 33075818 [12]	Fig. 2F WES; NGS-based copy number analysis Fig. 2G Custom NGS panel [d]; NGS-based copy number analysis
p.(Gln259Lys)	c.775C>A	0.00003096 (NFE)	0.1519	RCV000697732.3 VUS (Invitae) [¥]		BS3 supporting	BS3 supporting	VUS	VUS	PMID 20955399 (JMML patient) [13]	

[§] Our data provide evidence for the applicability of PS3 supporting for His58Asn, but other studies demonstrate functionality of this variant [3], so we suggest no application of functional data due to conflicting results. [#] PS3 and PVS1 cannot be combined. [¥] Classifications from the submitter *Invitae* are based on general ACMG/AMP guidelines, but do not follow RUNX1-specific MM-VCEP recommendations. [a] Custom NGS panel incl. *ANKRD26*, *ETV6*, *RUNX1*, *CYCS*, *SLFN14*, *DDX41*, and *STX11*. [b] Custom NGS panel for myeloid malignancies incl. *ASXL1*, *BCOR*, *BCORL1*, *CALR*, *CBL*, *CSF3R*, *DNMT3A*, *ETV6*, *EZH2*, *FLT3*, *GATA2*, *IDH1*, *IDH2*, *JAK2*, *KIT*, *KRAS*, *MPL*, *NIPBL*, NPM1, *NRAS*, *PHF6*, *PTPN11*, *RAD21*, *RIT1*, *RUNX1*, *SETBP1*, *SF3B1*, *SMC1A*, *SMC3*, *SRSF2*, *STAG2*, *TET2*, *TP53*, *U2AF1*, *WT1*, and *ZRSR2*. [c] Custom NGS panel for thrombocytopenia incl. *ABCG5*, *ABCG8*, *ACTN1*, *ANKRD26*, *AP3B1*, *AP3D1*, *BLOC1S3*, *BLOC1S6*, *CYCS*, *DIAPH1*, *DTNBP1*, *ETV6*, *FERMT3*, *FLI1*, *FLNA*, *FYB1*, *GATA1*, *GFI1B*, *GNAS*, *GP1BA*, *GP1BB*, *GP6*, *GP9*, *HOXA2*, *HPS1*, *HPS3*, *HPS4*, *HPS5*, *HPS6*, *ITGA2B*, *ITGB3*, *LYST*, *MECOM*, *MPIG6B*, *MPL*, *MYH9*, *NBEAL2*, *P2RY12*, *PLAU*, *PRKACG*, *PTPN11*, *RAB27A*, *RASGRP2*, *RBM8A*, *RGS18*, *RUNX1*, *SALL4*, *SLFN14*, *SRC*, *STIM1*, *TBXA2R*, *TBXAS1*, *TPM4*, *TRPM7*, *TUBB1*, *VIPAS39*, *VPS33B*, *VWF*, and *WAS*. [d] *ACBD5*, *ACD*, *AK2*, *ANKRD26*, *AP3B1*, *ATR*, *BRCA1*, *BRCA2*, *BRIP1*, *CDAN1*, *CDIN1*, *CSF3R*, *CTC1*, *CXCR4*, *DKC1*, *DNAJC21*, *ELANE*, *EPO*, *ERCC4*, *ERCC6L2*, *ETV6*, *FANCA*, *FANCB*, *FANCC*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCL*, *FANCM*, *G6PC3*, *GATA1*, *GATA2*, *GFI1*, *HAX1*, *HOXA11*, *JAGN1*, *KIF23*, *KLF1*, *LYST*, *MAD2L2*, *MASTL*, *MECOM*, *MPL*, *MYSM1*, *NAF1*, *NBN*, *NHP2*, *NOP10*, *PALB2*, *PARN*, *RAD51*, *RAD51C*, *RBM8A*, *RPL11*, *RPL15*, *RPL17*, *RPL18*, *RPL19*, *RPL26*, *RPL27*, *RPL31*, *RPL35*, *RPL35A*, *RPL5*, *RPS10*, *RPS15A*, *RPS17*, *RPS19*, *RPS20*, *RPS24*, *RPS26*, *RPS27*, *RPS28*, *RPS29*, *RPS7*, *RTEL1*, *RUNX1*, *SAMD9*, *SAMD9L*, *SBDS*, *SEC23B*, *SLC37A4*, *SLX4*, *SRP54*, *SRP72*, *TAZ*, *TBXAS1*, *TCIRG1*, *TERC*, *TERT*, *THPO*, *TINF2*, *TSR2*, *UBE2T*, *USB1*, *VPS13B*, *VPS45*, *WAS*, *WRAP53*, *XRCC2*, *ADA2*, *COP1*, *EFL1*, and *STN1*. Data from functional assays were included, regardless of the underlying nucleotide change, except for variants, which might affect splicing. EA – East Asian; LB – likely benign; NFE – Non-Finish European; NGS – next generation sequencing; w/o – without; w/ - with; TA – transcriptional activation assay; VUS – variant of uncertain significance; WES – whole exome sequencing.



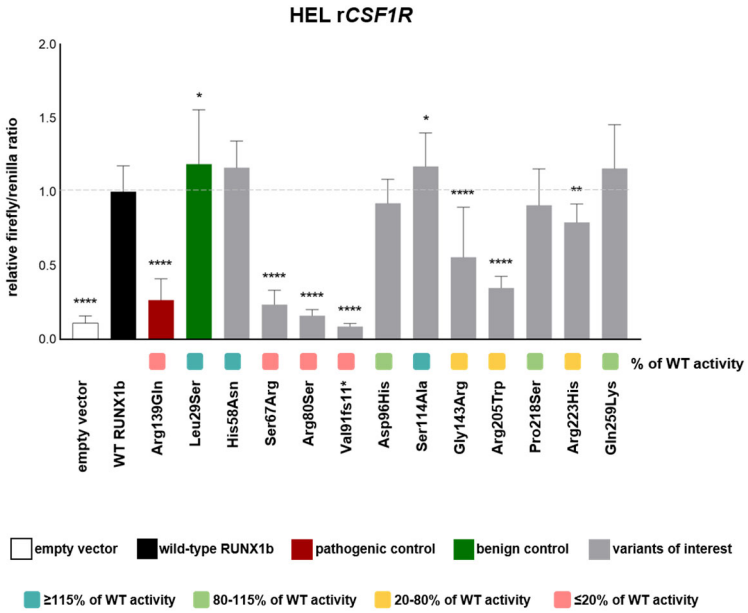
Supplementary Figure 1. Transcriptional activation assay r*CSF1R* in HEK293T analyzing the set of RUNX1b variants of interest.

The bar graph displays firefly/renilla ratios relative to wild-type RUNX1b (WT) (mean+ standard deviation (SD); 3 biological and 6 technical replicates; one-way ANOVA in comparison to WT; Dunnett's post hoc test; *, P ≤.05; **, P ≤.01; ***, P ≤.001; ****, P ≤.0001).

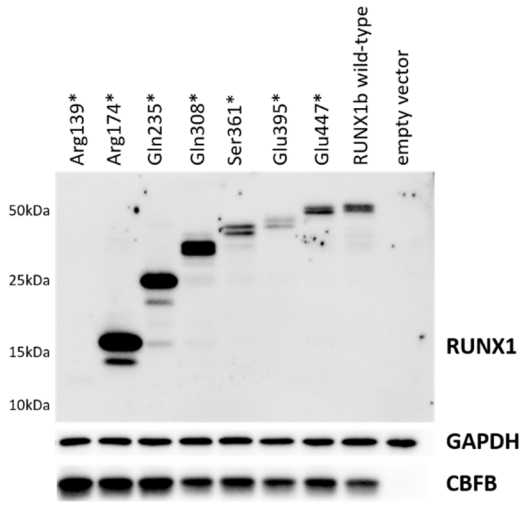


Supplementary Figure 2. Transcriptional activation assay r*MYL9* in HEK293T analyzing the set of RUNX1b variants of interest.

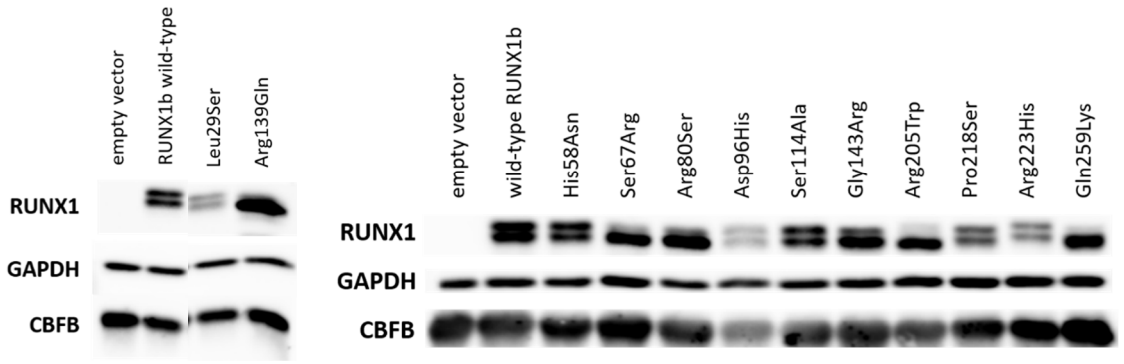
The bar graph displays firefly/renilla ratios relative to wild-type RUNX1b (WT) (mean+SD; 3 biological and 6 technical replicates; one-way ANOVA in comparison to WT; Dunnett's post hoc test; *, P ≤.05; **, P ≤.01; ***, P ≤.001; ****, P ≤.0001).



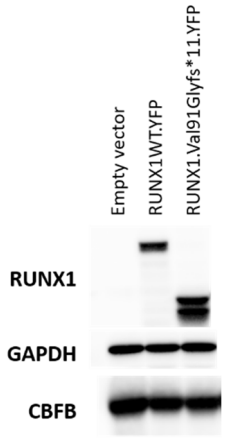
Supplementary Figure 3. Transcriptional activation assay r*CSF1R* in HEL analyzing the set of RUNX1b variants of interest. The bar graph displays firefly/renilla ratios relative to wild-type RUNX1b (WT) (mean+SD; 2 biological and 5 technical replicates; one-way ANOVA in comparison to WT; Dunnett's post hoc test; *, P ≤.05; **, P ≤.01; ***, P ≤.001; ****, P ≤.0001).



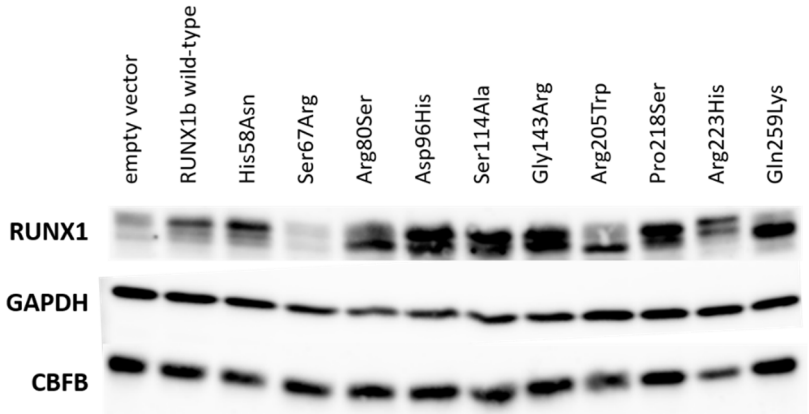
Supplementary Figure 4. Representative Western Blot analyzing transfection conditions used for transactivation assays of missense variants in HEK293T cells. Transfection and Western Blotting was performed as previously described [14]. The truncated protein variant RUNX1b-Arg139* was too small to be clearly detected using standard conditions. Under these conditions, only a very slight band could be observed. However, the overexpression of Arg139* and an YFP-tagged Arg139* fusion protein has already been demonstrated by real-time PCR and western blotting, respectively [14].



Supplementary Figure 5. Representative Western Blot analyzing transfection conditions used for transactivation assays of VOI in HEK293T cells. Transfection and Western Blotting was performed as previously described [14].



Supplementary Figure 6. Representative Western Blot verifying the expression of RUNX1.Val91Glyfs*11 in HEK293T cells. As the resulting truncated RUNX1 protein is too small to be detected under standard western blot conditions, we analyzed the YFP-tagged WT and YFP-tagged variant RUNX1 protein as previously described [14].



Supplementary Figure 7. Representative Western Blot analyzing transfection conditions used for transactivation assays in HEL cells. Transfection and Western Blotting was performed as previously described [14].

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